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"EFFECT OF SMA-DMSO GNP ON CELL SURVIVAL AND DNA DAMAGE ANALYSIS IN MCF-7 & HEK CELL LINE BY MTT AND COMET ASSAY"

AVINASH SINGH[1], CHAITI GANGULY[2] & R. K. SINGH[3]

[1,2]Department of Biotechnology, CET, IILM – AHL, Greater Noida, Uttar Pradesh, India

[3]CSIR-Central Drug Research Institute, Lucknow Uttar Pradesh, India

ABSTRACT

Male hormonal and non-hormonal contraceptives have been subject of great interest at present at global level. SMA-DMSO complex is one such non-hormonal male contraceptive compound which has been extensively studied by many researchers and found promising results. Use of such SMA-DMSO GNP Compound has been found to contain some adverse cyto-toxic and carcinogenic effect as well. However some research reports have also reported the decreased toxic effect of SMA-DMSO after encapsulating the compound SMA-DMSO GNP.

Present study aims to study the effect of different dosage of SMA-DMSO GNP on cell survival and DNA damage. MCF 7 and HEK cell viability and DNA damage has been tested against SMA-DMSO GNP in the dosage range of 25 nM to 200 nM using MTT and COMET assay at different time period of 24 hr to 48 hr.

The result shows that SMA-DMSO GNP significantly increases the survival of cell line MCF 7 & HEK cell lines. Along with DNA damage was also found negligible in different time period by comet assay test. The following study may conclude that SMA-DMSO GNP have better chance of application as contraceptive solution to men's population. However further interrogative assays need to be carried to assess its genotoxic potential, allergenicity & carcinogenicity.

KEYWORDS: SMA-DMSO, SMA-DMSO GNP, MTT, COMET, MCF 7 & HEK

INTRODUCTION

The internalization of compound SMA-DMSO GNP is assess on the basis of internalization of anticancer herbal droug[1,9,10], and the pharmacokinetics [2]. The determination of cellular toxicity is based on tetrazolium-based assays. The tetrazolium based assay are probably the most widely used tests[3].. In 1963, Slater et al. reported for the first time that tetrazolium salts were reduced by the mitochondrial respiratory chain [3]. In 1983, Mosmann came up with the idea that this property could be used to measure cellular proliferation and survival, and developed the MTT assay [4]. Later studies however demonstrated that most of the MTT reduction occurred at extra mitochondrial sites, involving pyridine nucleotides NADH and NADPH [34]. Besides MTT, several other dyes such as XTT, MTS, or WST-1 may be used. These present the advantage of carrying a negative charge that allows them to remain soluble after reduction, avoiding the formazan solubilization step [6]. In cell-based systems, it appears mandatory to carefully wash the cells to remove the maximum of potentially interfering phytochemicals before adding the tetrazolium dye. Brugisser et al. have shown that the flavonoid kaempferol could directly reduce MTT; its effects were limited when the incubation medium was discarded and cell cultures washed properly [7-9,11,12]. But washing operations obviously will not be efficient for strongly adsorbed or internalized compounds. Tetrazolium tests may also be influenced by modulations in mitochondria amounts and activities.

For example, the flavonoid genistein induces a G2/M cell cycle arrest followed by cell death in tumor cells The quantitative estimation of cell survival by using MTT assay (21)

As per the guidelines of ICH ( ), minimal 3 test battery needs to be conducted in order to assess the mutagenicity and toxicity of compounds which includes AMES test in bacterium strain, chromosomal damage test in mammalian cell line under invitro or invivo conditions and Sister chromatid assay (19)followed by Dominant lethal test (20) in invivo conditions. Comet Assay is a prominent methodology to assess DNA damage in cell lines. Comet Assay is carried in alkaline and normal mode to verify the effect of test chemicals on the alkaline labile sites of DNA.Single strands DNA breaks are analysed using alkaline COMET and double stranded breaks are analyzed using neutral COMET (20). As per the standardized protocol of Comet assay, individual cells embedded in agarose are lysed and than subjected to electrophoresis. Fluorescence microscopy along with staining with PI helps in the visualization of denatured DNA fragments migrating out of the cell nucleus. The image obtained is a "comet" with a distinct head consisting of intact DNA and a tail containing relaxed DNA loops or broken pieces of DNA [108]. DNA percentage in Tail including the intensity of the tail along with measurement of Olive tail movement gives a substantial measure of DNA damage and any cross linking induced by the test chemical.

Throughout the years, COMET assay has proved to be an effective methodology to investigate all kind s of DNA damages comprising of Oxidative damage, alkylation effects, strand breaks, formation of bulk adducts etc. Applicability of this assay has enhanced in genotoxicity measurement due to ease of application and high accuracy of results.(18)

METHODOLOGY

Media

Media used for culturing of the above cell line were L-15 Medium with L-Glutamine and FBS (Fetal Bovine Serum). Some serum free media also used provided by, Hyclone). To check the any type of bacterial or fungal contamination, used Thioglycollate medium, Tryptone soya broth.

Cell lines

Cell lines purchased from NCCS pune and used for evaluation of toxicity by MTT and DNA damage by COMET Assay: The HEK and MCF 7 cell lines were used.

The inverted microscope was used for visualization of growth of cells and also monitors the absence of bacterial and fungal contaminants. The growth of the cells was in monolayer and the monolayer of the cells was washed with PBS without Ca2+/Mg2+ in equal volume of culture medium. After washing cell monolayer, the cells was detached by adding of Trypsin/EDTA and incubate in incubator for 2-10 minutes. The detached cells were floating in suspended medium and it was observed by inverted microscope. The density of the cells was calculated by hemocytometer. Now the sub-culturing was performed in the above medium contains SMA-DMSO GNP.

MTT Assay, Cell Proliferation, Survival & Cell Culture

The HEK and MCF 7 cells are more easy in maintaining and easily internalize the SMA DMSO GNP. The HEK cells was cultured in Leibovitz L-15 medium containing L Glutamine and FBS and MCF 7 cells in SFM HEK (Serum Free Media, Hyclone),. Cells in medium containing SMA-DMSO GNP were incubated in CO_2 incubator (Thermo Scientific Make) at 37^0C with a regular change of the medium at an interval of 5 days. The cell viability were monitored on

a regular basis by using vital dye like as a tetrazolium.

The actively growing cells release the enzyme dehydrogenase which reduced the dye(tetrazolium MTT) resulting in intracellular purple formazan which is quantified by spectrophotometer. In this study the high cell density were plated and incubated (at 37^0C) for the period of 24 hrs. to 48 hrs. After that add 10 µl MTT reagent (Sigma Aldrich) (12 mM stock concentration) and incubated till the appearance of purple precipitates (2 to 4 hrs). In the dark room at $27^0C \pm 2^0C$, 100 µl detergent reagent was added in each microtitre plates and left for 2 hours. Absorbance was taken and recorded at 570 nm by using ELISA plate reader. The test compound were added in plates (SMA-DMSO & SMA-DMSO GNP) at different concentrations to study the response of the cells for these compounds. Prepared SMA-DMSO was provided by the CSIR-CDRI laboratory Lucknow , SMA DMSO GNP was prepared in IILM Gr Noida and other chemicals were provided by the Hi-Media Inc.Hyclone.

The Cell viability of the cells was calculated by hemocytometer, the unstained cells are viable

Percentage of Cell Viability = (Total viable cells / Total cells) × 100

Further I was checked the Cytotoxicity

Further the cytotoxicity was calculated by taking the absorbance at 570 nm

Cytotoxicity = (A –B)/ (C-B)

Where A) is OD at 570 nm obtained from a well added with a test chemical; B) is the mean OD at 570 nm of blank wells; and C) is the mean OD at 570 nm derived from control wells (added culture medium as a test sample).

Statistical Analysis

Experiments were carried in triplicates of each set. The optical density values were statistically analyzed using Microsoft Excel sheets.

Low melting point agarose (LMPA), Ethidium bromide (EtBr), Triton-X-100, Ethyl methanesulfonate (EMS, CAS No: 62-50-0) and Trypan blue were procured from Sigma Chemicals Co.Ltd., St.Louis, MO, USA. Normal melting agarose (NMA), Ethylenediaminetetraacetic acid disodium salt, MTT, Trypsin-EDTA, Fetal bovine serum (FBS) were procured from Hi Media Pvt. Ltd. Mumbai, India. Phosphate buffered saline (Ca^{2+}, Mg^{2+} free; PBS), Ham's F12 medium Antibiotic and antimycotic solution (10,000 U/ml penicillin, 10 mg/ ml streptomycin and 25 µg/ ml amphotericin B) were obtained from Life Technologies (India) Pvt. Ltd. New Delhi, India. And the other chemicals like DMSO, NaCl and NaOH were purchased from Merk Pvt. LTd

Cell Culture and Treatment

Human Breast Adenocarcinoma (MCF-7) and HEK cells was purchased from National Centre for Cell Sciences, Pune, India and maintained in antibiotic containing DMEM with supplement medium 10% FBS .The cells were inoculated in animal cell culture flasks at 37^oC and incubate in CO_2 incubator.

MCF7 and HEK cells were detached using 1.5 ml of 0.25% trypsin and when all the cells detached, trypsin was detoxified with 2 ml complete medium. The growned cells were separated by help of centrifuge at 1000 rpm for 10 minutes and the cells pellet was resuspended in complete medium to give 10^6 cells / ml solution. Once I was obtained the cell

density approximately 10^6 cells / ml solution, then seeded in microtitre plate (10,000 cells per well) for check the viability by MTT assay and DNA damage by Comet assay.

Treatment preparation

25 nM to 200 nM of SMA-DMSO was taken in a 50 ml centrifuge tube and the volume was made upto 8 ml with DMEM serum free medium (IF12) and sonicated at 14 MHz till clear solution was obtained. The sterilized solution was obtained by using 0.22 mm filter and adjust the pH 7.5 and the final volume was made 10 ml by adding IF12 medium. and filter sterilized through 0.22mm filter. After following the above procedure the prepared solution contains 1 mM concentration of SMA-DMSO. From the above solution (stock solution) different concentrations were made from stock solution by appropriate dilution.

In the SMA DMSO Complex treated MCF-7 cells, the single cell gel elctrophoresis (comet assay) shows the concentration and time dependent DNA damage. At 50nM concentration DNA fragmentation was observed 12 hour post treatment while at 25nM the fragmentation was observed 24 hour post treatment. Void nanoparticles do not show any DNA fragmentation. At 25nM PLGNP, DNA fragmentation was observed 24 hour post treatment but at 50nM DNA fragmentation was delayed & was only observed at 48 hours post treatment. The extent of DNA fragmentation was explained in the form of scores that were calculated from the content of fragmented DNA & their migration, by electrophoresis (figure 6, Table 2).

RESULTS AND DISCUSSIONS

Cellular Internalization of Nanoparticles

For understanding the internalization of nanoparticles cellular uptake was assessed in a fluorescence microscope. MCF-7 & HEK cells were treated with FITC labeled gelatin nanoparticles. The cells were fixed visualized I microscope (Eclips 90i, Nikon). It is possible that Nanoparticles(NP) may be taken up by cells via their size selectivity that may match those of endocytic pits. NP endocytosis does not only depend on size by of nano-particles, but also on the surface coating and charge.

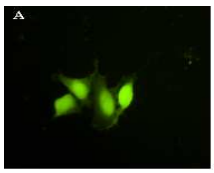


Figure 1: Internalization of FITC Labeled Gel NP's by MCF-7 (40x) (A). **Since the Nucleus of the Cells was Stained by Propidium Iodide (PI), in (B) the Yellow Colour Confirmed the Internalization of Nanoparticle in Cytoplasm and Deported to Nucleus Also**

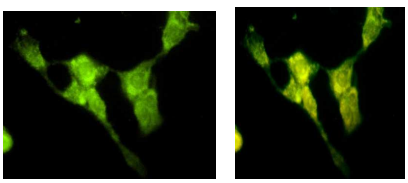
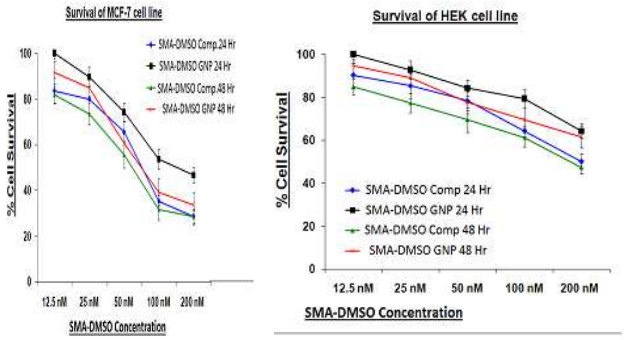


Figure 2: Internalization of FITC Labeled Gel NP's By HEK (40 xs) (A). Since The Nucleus of the Cells was Stained by Propidium Iodide (PI), in (B) the Yellow Colour Confirmed the Internalization of Nanoparticle in Cytoplasm and Deported to Nucleus Also but in Less Quantity as the Intensity of Yellow Colour was Less

Effect of SMA DMSO Complex Entrapment on Cell Survival



(a) (b)

Figure 3: The cell survival of (a) MCF-7 & (b) HEK Cell Line on the various Concentration of SMA DMSO Complex & SMA DMSO GNP at Different Time Point

The cytotoxicity of SMA DMSO Complex *per se* and SMA DMSO GNP was assessed on both the cell line MCF7 and HEK. The survival of cells was inversely proportional to the dose of SMA DMSO Complex but 80% survival was observed with the same dose of SMA DMSO Complex entraped in nanoparticles (SMS DMSO GNP). With the increase in concentration of SMA DMSO Complex from 25nM to 200nM the survival on MCF-7 decreased from ~85% to 49% in 24 hr (figure 3a) and on HEK-29 it decreased from ~78% to 40% in 24 hr (figure 3b). But after entrapment in nanoparticles the cell survival increased by ~ 10-30% in both the cell lines.

Nanoparticle Modulate ROS & RNS Production

O Al-alami *et al* (1998). has reported that a significant increase in nitric oxide production during Paclitaxel - induced apoptosis in U937 monocytic leukaemia cells, confirming the vital role of nitric oxide in mediating paclitaxel-induced apoptosis by monocytic cells. A number of gross biochemical changes occur as a consequence of oxidative stress. Oxidative stress can elicit cell death, and that mild oxidative stress can initiate apoptosis rather than necrosis. Although reactive oxygen species can cause oxidative stress, they are not essential for the apoptotic processes to occur (S. Glutton, 1997). Interleukin 1β converting enzyme (ICE) - family proteins play the part of cellular executioner, but the biochemical messengers yet remain to be determined.

In our studies, we have found that elevated levels of RNS (NO) & ROS (TPA) are directly proportional to cytotoxicity with SMA DMSO Complex treated cells in a dose as well as time dependent manner. But **after entrapment of drug in gelatin nanoparticles cytotoxicity as well as ROS & RNS decreases**. So, the SMA-DMSO GNP prevents the cells from the oxidative DNA damage and cytotoxicity. With the increase in concentration of SMA DMSO Complex from 25nM to 200nM the cytotoxicity on MCF-7 also increased and reached to 52% in 24 hr & 58% in 48 hour (figure 4). But the cytotoxicity caused by SMA-DMSO GNP was reduced to 37% in 24 hr and 42% in 48 hour. On HEK cells the cytotoxicity was 59% at maximum concentration of SMA DMSO Complex (200nM) in 24 hrs and 65%in 48 hrs, but after entrapment in NP's the cytotoxicity reduced to 42% in 24 hrs and 48% in 48hrs (figure 4).

TPA and NO production are also regulated by the SMA DMSO GNP and we found the concentration of TPA & NO was upregulated after the treatment of SMA DMSO Complex *per se*, on the other hand SMA DMSO Complex encapsulated nanoparticles downregulated the production of TPA & NO (Figure 4,5). In other words we observed down

regulation in the release of free radicals (H_2O_2 & NO) with the decrease in the cytotoxicity of cells post treatment with SMA DMSO GNP.

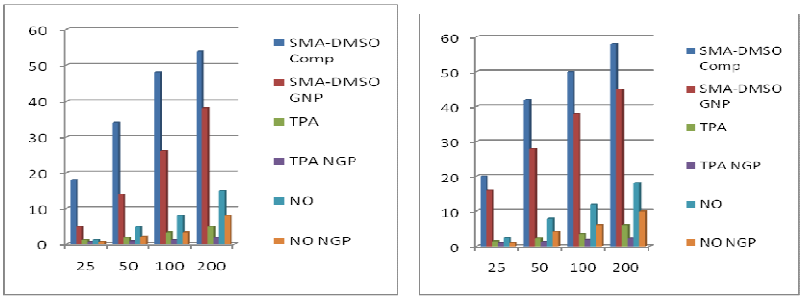


Figure 4: The Cytotxicity & ROS (TPA & NO) Release from the MCF-7 was Dose and Time Dependent (A) at 24hr Post Treatment and (B) 48 Hr of Post Treatment.(Positive Control 0.1MH_2O_2 = 84.96nm TPA)

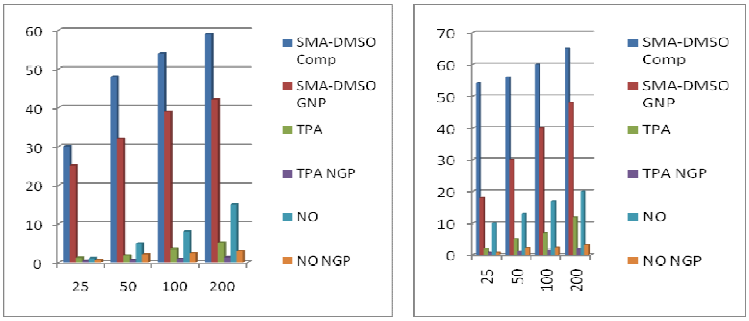


Figure 5: The cytotxicity & ROS (TPA & NO) Release from the HEK was Dose and Time Dependent (a) at 24hr Post Treatment and (b) 48 hr of Post Treatment. (Positive Control 0.1MH_2O_2 = 84.96nM TPA) with the Increase in Concentration of SMA DMSO Complex from 25nM to 200nM the Cytotoxicity also Increased from ~30% to 59% in 24 hr & 65% in 48 hour

Nanoparticle Prevent Nucleic Acid (DNA) Damage

Principle

The nucleic acid damage was analyzed by gel electrophoresis. The comet assay is generally used analysis of DNA damage at cellular level. It is a highly sensitive, non expensive and visual technique.

Treated cells are mixed with low melting agarose and layered onto conventional microscope slides. The cells are lysed using high salt concentration and the double DNA is subjected to highly alkaline conditions (pH 13) to relax into single strands and express alkaline labile sites, which under the influence of electric current stretch out from the porous nuclear membrane into the agarose gel. These strands of DNA upon neutralization (pH 7.5), form hairpin loops thereby facilitating intercalation of fluorescent DNA dye. The final image obtained looks like a “Comet” with a distinct head, comprised of intact DNA and a tail made of broken strands of DNA.

Table 1: Comet Score of DNA Damage

Samples ↓ Hours →	Comet Score			
	6 Hr	12 Hr	24 Hr	48 Hr
Control	0	0	0	0
Gelatin NP’s	0	0	0	0

Table 1: cond.,				
SMA DMSO Complex (25nM)	0	0	1	2
SMA DMSO Complex (50nM)	0	1	2	3
SMA DMSO GNP (25nM)	0	0	0	1
SMA DMSO GNP (50nM)	0	0	1	3

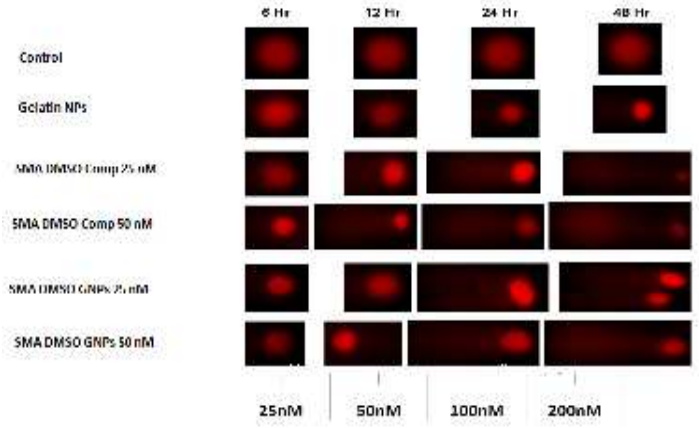


Figure 6: The Single Cell Gel Electrophoresis of MCF-7 & HEK Cells

DISCUSSIONS

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